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# The Effect of Indomethacin on Physical Activity in a Mouse Model of Cancer Cachexia

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Honor Thesis

6/5/2009

## Abstract

Fatigue decreases the quality of life for patients with cancer cachexia (McCarthy, 2003). Skeletal muscle wasting is a major contributor to the experience of fatigue. Indomethacin, an anti-inflammatory drug, has been shown to reduce skeletal muscle wasting in animal models of cancer cachexia (Hitt, Graves & McCarthy, 2004). The purpose of this study is to determine if indomethacin affects fatigue, measured as voluntary wheel running activity (VWRA) in mice bearing the colon-26 adenocarcinoma (C26). The hypothesis is that VWRA and muscle mass will be maintained in tumor-bearing mice given indomethacin in their food. Mice were divided into 4 groups, which had access to running wheels in their cages. Group 1 received neither tumor cells nor indomethacin, group 2 received indomethacin, group 3 received tumor cells and group 4 received tumor cells and indomethacin. A fifth group did not have access to running wheels and served as a control for the effect of VWRA on muscle mass in tumor-bearing mice. All mice were caged individually and VWRA was electronically monitored for seventeen days. The body weight and muscle weights were analyzed using two way analysis of variance. VWRA was analyzed using two way repeated measures analysis of variance. Tumor mass was analyzed using one way analysis of variance.

Indomethacin proved to be toxic. There was a main effect of indomethacin ($p= 0.03$; $f=5.2$) and a main effect of tumor ($p= 0.03$; $f=5.4$) on VWRA. Muscle mass was significantly reduced by tumor ($p= 0.014$; $f=7.1$), but it was not affected by indomethacin. Our hypothesis was not supported: indomethacin did not maintain muscle mass or VWRA in tumor bearing mice. However, tumor mass was significantly smaller in tumor-bearing mice in group 4 compared to group 5 and 3. Further study is needed to determine if other anti-inflammatory drugs would be less toxic than indomethacin in this animal model of cancer associated fatigue.

## Introduction

Fatigue is the symptom most frequently reported by cancer patients (Al-Majid & McCarthy, 2001). One factor that contributes to fatigue is skeletal muscle wasting, which occurs in cancer patients with cancer cachexia. Cancer cachexia, a syndrome characterized by decreased body weight, anorexia, decreased muscle mass, weakness and fatigue, is a prevalent and serious problem in cancer patients (Skipworth, Stewart, Dejong, Preston, & Fearon, 2007). The exact mechanism by which cancer cachexia occurs is still unknown. In the following study, a mouse model will be used to investigate fatigue in mice bearing the colon-26 adenocarcinoma (C26). Volunteer wheel running activity (VWRA) will be monitored as an indication of fatigue. Muscle mass will also be measured in response to tumor growth and treatment with indomethacin. Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) that has been shown to preserve muscle mass in mouse models of cancer cachexia (McCarthy, Whitney, Hitt, & Al-Majid, 2004; Hitt, Graves, & McCarthy, 2004) and will be administered in mouse chow.

There were 4 groups of mice with access to running wheels: Group 1 is the control group and did not receive the tumor cells or indomethacin. Group 2 received indomethacin, but not the tumor cells (to control for the effects of indomethacin). Group 3 received the tumor cells, but not indomethacin (to control for the effects of tumor growth). Group 4 received both indomethacin and the tumor cells. A fifth group received tumor cells, but did not have access to running wheels. This group served as a control for the effects of wheel running on muscle mass in tumor-bearing animals. The research question of interest is: will indomethacin, which has previously been shown to preserve muscle mass in tumor-bearing mice, improve voluntary wheel running activity in tumor-bearing mice. The hypothesis to be tested is that VWRA and muscle

mass will be maintained in tumor-bearing mice (as compared to the non-tumor control mice) given indomethacin in their food, compared to those mice who did not receive indomethacin. The overall goal of this program of study is to reduce fatigue related to muscle wasting and thus increase the quality of life for patients with cancer cachexia.

## Review of Literature

Cachexia can be described as a chronic syndrome characterized by skeletal muscle wasting, anorexia, weight loss, weakness and fatigue (Skipworth, Stewart, Dejong, Preston, & Fearon, 2007). It is associated with altered metabolism of protein, glucose, and fat. Between 50% and 80% of cancer patients develop cancer cachexia in the advanced stages of cancer, and it is a significant cause of morbidity and mortality (McCarthy, 2003). Studies have found that patients with cancer cachexia report lower quality of life scores and reduced physical activity (Skipworth, et al., 2007). Cancer cachexia is a syndrome that affects both the patients' physical and psychosocial wellbeing. Therefore, interventions to reduce fatigue in patients with cancer cachexia would increase their quality of life.

Significant skeletal muscle wasting occurs in approximately 50% of patients with cancer cachexia (Al-Majid & McCarthy, 2001). Skeletal muscle wasting is a result of a decreased rate of skeletal muscle protein synthesis and an increased rate of skeletal muscle protein breakdown. Decreased muscle protein synthesis is a result of several factors. For example, protein synthesis entails the activation of the signaling pathway that results in the phoshorylation of p70, a ribosomal kinase that is involved in the translation mRNA into protein (Baar & Esser, 1999; Glass, 2003). A study found that phoshorylation of p70 was reduced in the gastroc muscle of mice bearing the colon26 adenocarcinoma (McCarthy, et al., 2004). Tumor growth also triggers

the neuro-endocrine stress response; elevated serum cortisol levels and insulin resistance are associated with reduced muscle protein synthesis (Skipworth, et al., 2007).

Skeletal muscle protein breakdown is influenced by several factors as well. The tumor induces the release of local pro-inflammatory cytokines such as interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα) and interferon-gamma (IFNγ). These cytokines produce anorexia, proteolysis in skeletal muscle, and alter the metabolism of carbohydrates, lipids and proteins (McCarthy, 2003). In addition, the release of pro-cachectic factors such as proteolysis inducing factor (PIF) and lipid mobilising factor (LMF) can cause catabolism of host tissues (Skipworth, et al., 2007). These changes lead to the loss of skeletal muscle mass.

Studies have shown increased activity of the calcium/calpain pathway and the ubiqutin-proteasome pathway (UPP) in skeletal muscle breakdown (Skipworth & Fearon, 2007). The UPP is thought to breakdown contractile skeletal muscle proteins through complex signaling pathways involving the upregulation of the UPP (Attaix, et al., 2005). The protein is polyubiquitinated and recognized by the 26 S proteasome that then breaks down the protein into free amino acids (Attaix, et al., 2005). Studies have shown that IL-6 and TNFα increase the activity of the UPP (McCarthy & Graves, 2006). Also, when skeletal muscle is broken down into the free amino acids they are used in the liver for synthesis of acute phase proteins such as CPR. Increased serum CPR levels are positively correlated with weight loss, anorexia, hypermetabolism, the extent of the disease and reduced survival time in cancer patients (Skipworth & Fearon, 2007).

There is growing evidence that the cyclooxygenase (COX) pathway plays a role in increased skeletal muscle protein breakdown (McCarthy & Graves, 2006). Arachidonic acid is a twenty carbon polyunsaturated fatty acid found in the phospholipid layer of the cell membrane. Arachidonic acid is converted by COX and lipoxygenase (LOX) enzymes into prostaglandins and leukotrienes (McCarthy, 2003). COX has two isoforms, COX-1 and COX-2. COX-1 is normally produced to maintain homeostasis in the body and COX-2 is produced in response to infection or inflammation. PGE2, a pro-inflammatory prostaglandin, augments the production of IL-1, IL-6 and TNFα in macrophages. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the activity of COX to decrease inflammation (McCarthy, 2003). Increased plasma PGE2 levels and increased expression of the COX-2 has been found in animal models of cancer cachexia (McCarthy, Whitney, Hitt, & Majid, 2004). Many different tumor lines have been reported to express increased levels of COX-2 (Cao & Prescott, 2002). This has increased the amount of research studying the effect of NSAIDs on tumor growth.

Research has indicated that NSAIDs reduce tumor growth in vitro by initiating cell cycle arrest and increasing apoptosis (programmed cell suicide) (Eli, Przedecki, Levin, Kariv, & Raz, 2001; Cao & Prescott, 2002; Cheng, Imanishi, Amuro, & Hada, 2002; Raz, 2002). Tumor-bearing mice treated with NSAIDs have reduced tumor mass, reduced blood vessel growth in the tumor bed and reduced incidence of metastasis (Liu, Kirschenbaum, Yao, Lee, Holland, & Levine, 2000; Masferrer, Leahy, Koki, Zweifel, Settle, Woerner, Edwards, Flickinger, Moore, & Seibert, 2000; Kundu, Yang, Dorsey, & Fulton, 2001; Kundu & Fulton, 2002). It has also been discovered that compared to the control group, decreased tumor mass in mice treated with NSAIDs is associated with an increase in survival time, preservation of body weight and muscle

mass (Hussey & Tisdale, 2000; Ross & Fearon, 2002). Further research is needed to determine if NSAIDs reduce muscle wasting independently of tumor growth.

Research has also demonstrated that NSAIDs slowed weight loss and prolonged survival time in cancer patients (Lundholm, Gerlin, Hytlander, Lonnroth, Sandstrom, & Svaninger, 1994; Lundholm, Daneryd, Korner, Hyltander, & Bosaeus, 2004; Deans & Wigmore, 2005). Other studies using an animal model of cancer cachexia reported decreased weight loss, increased muscle mass, and reduced serum TNFα and IL-6 levels in animals treated with indomethacin, a NSAID that inhibits the activity of both COX-1 and COX-2 pathways as well as the LOX pathway (Cahlin, Korner, Axelsson, Wang, Lunddholm, & Svanberg, 2000; Hussey & Tisdale, 2000; Zhou, et al. 2003; Davis, Zweifel, O'Neal, Heuvelman, Abegg, & Hendrich 2004; McCarthy, 2003).

Cancer cachexia causes skeletal muscle wasting that leads to asthenia and fatigue, which can therefore alter the patients' physical capabilities (Al-Majid & McCarthy, 2001). This restriction has caused fatigue to be considered the most upsetting symptom in these patients (Al-Majid & McCarthy, 2001). Decreased physical activity leads to disuse atrophy and thus compounds the sensation of fatigue. Research has shown endurance exercises decreases the patients' level of fatigue (self reported) and increase activity tolerance in patients with cancer (Al-Majid & McCarthy, 2001).

VWRA is a normal activity for caged mice and is a model of fatigue in mice (Ottenweller, Natelson, Gause, Carroll, Beldowicz, Zhou, & Lamanca, 1998). In one study, VWRA was found to be a more sensitive measure of sickness behavior than grooming behavior in mice and could be used as a model to study the relationship between fatigue and immune activation (Ottenwellwer, et al., 1998). Both tumor growth and chemotherapy (V-16) have been

shown to decrease VWRA in mice, and the decrease in VWRA is greatest in tumor-bearing mice (Wood, Nail, Perrin, Elsea, Fischer, & Drunker, 2006). Overall, a decrease in VWRA is to be inferred as fatigue in rodent models of sickness behavior (Cho, DeLaHunt, Hu, Close, & Peterson, 1992; Ottenweller, et al., 1998; Sheng, Hu, Lamkin, Peterson, & Chao, 1996).

The purpose of this study is to examine VWRA in tumor-bearing mice treated with indomethacin. We hypothesize that tumor-bearing mice treated with indomethacin will have increased voluntary wheel running and muscle mass compared to the control mice.

## Methods

Female CD2FI (BALB/c x DBA/2) pathogen free mice were housed individually in cages modified to include the activity wheel and maintained on a twelve hour light dark schedule that commenced at six a.m. A separate group of mice were maintained in individual cages without access to an activity wheel. All mice had free access to water and ground chow at all times. Ground rodent chow was administered in spill proof glass dishes. The food intake was monitored by subtracting the weight of the remaining food from the original weight that was placed in their cages. Mice were weighed at the beginning and at the end of the experiment using the same scale.

The mice in activity cages were given three to five days to establish their baseline levels of VWRA. VWRA was continuously measured using a magnetic switch that is interrupted by the side ports of the wheel (twice for each turn of the wheel). The interruptions were recorded in 2-minute intervals using a Mini Mitter Magnetic Switch and the Vital View Data Acquisition System.

There were five groups of mice in this experiment, the first four of which had access to running wheels; group 1 is the control group and did not receive the tumor cells (C26) or

indomethacin. Group 2 received indomethacin, but not the tumor cells to control for the effect of indomethacin on muscle mass and VWRA. Group 3 received the tumor cells, but not indomethacin to control for the effect of tumor growth on muscle mass and VWRA. Group 4 (the experimental group) received both indomethacin and the tumor cells. Group 5 was the sedentary tumor-bearing group to control for the effect of VWRA on muscle mass.

The murine colon-26 adenocarcinoma cell line was maintained in culture and passed using trypsin when confluent as previously described elsewhere (Al-Majid, & McCarthy, 2001). Mice in groups 3, 4 and 5 received subcutaneous inoculation of $5x\ 10^5$ tumor cells in 0.2mL of saline between the scapulae. The colon-26 adenocarcinoma cell line should produce a palpable mass within 7-10 days (Al-Majid, & McCarthy, 2001). The tumor does not metastasize, and the growth of the tumor is not associated with a decline in food intake as established in previous studies (Al-Majid, & McCarthy, 2001; Diffee, Kalfas, Al-Majid, & McCarthy, 2002; Zhou et al., 2003). After injection of the tumor cells, mice in group 2 and group 4 received the indomethacin in their food. Indomethacin was mixed into the ground rodent chow using a small blender with 0.015mg per gram of food. A 20g mouse normally eats 3-5grams of food per day, which would provide a maximum intake of 2.5mg/kg/day indomethacin respectively.

Mice were maintained on this diet for seventeen days and then were euthanized by inhalation of carbon dioxide followed by cervical dislocation. Each mouse was weighed and the gastrocnemius muscle of both limbs was removed, weighed, wrapped in foil, and stored on dry ice. The tumor was removed and weighed as well.

Data analysis was completed using SPSS version 16. The body weight and muscle weights were analyzed using two way analysis of variance for effects of tumor growth and drug. VWRA was analyzed using two way repeated measures analysis of variance. Tumor growth was

analyzed using one-way analysis of variance with the Duncan procedure for post hoc pairwise comparisons.

## Results

This experiment was run three times and 36 mice were originally included in the study. The dose of indomethacin, 2.5mg/kg/day proved to be toxic to the mice in the activity cages. Data from 2 mice were excluded due to premature death. Data from a third mouse that did not run was also excluded. Data from 33 mice were analyzed. Group 1 (no tumor no drug) N = 9, group 2 (no tumor and drug) N = 5, group 3 (tumor no drug) N = 8, group 4 (tumor and drug) N = 5 and group 5 (tumor sedentary) N = 6.

The first research question addressed in this study was: Does indomethacin preserve muscle mass? Using two way analysis of variance, this study found that there was a significant effect of tumor on muscle mass ($p=.014$; $f=7.1$). However, there was no significant effect of drug on muscle mass (see figure 1).

The second research question was: Does indomethacin preserve voluntary wheel running activity? VWRA was divided into six 3-day periods, starting with 3 days before injection of tumor cells. All mice started running with the commencement of the dark phase of their circadian rhythm. However, the total amount of VWRA in tumor-bearing animals declined over the next 15 days compared to the control animals, and the amount of VWRA declined in both the tumor and control animals given indomethacin (see figure 2). Using two way repeated measures analysis of variance, there was a significant main effect of tumor ($p=.03$; $f=5.4$) and drug ($p=.03$; $f=5.2$) on VWRA during the dark cycle (see figure 2). There was no interaction between the tumor and the drug on VWRA, meaning that the drug had the same effect on VWRA in the tumor bearing animals and control animals.

Since our data did not support our hypothesis that indomethacin would preserve muscle mass and VWRA, we examined correlations between tumor weight, muscle mass and VWRA during the last three days of the experiment. There was a significant negative correlation between tumor mass and VWRA ($r = -0.5$; $p=.01$). There was a significant positive correlation between average gastroc weight and VWRA ($r = 0.65$; $p=.01$). Also, in the three groups of tumor-bearing mice, tumor weight is negatively correlated with muscle mass ($r = -0.55$; $p=.03$).

One-way ANOVA was used to examine differences in tumor weight in the 3 groups of tumor-bearing mice. There was a significant difference between the tumor weight in wheel running mice, wheel running mice given indomethacin and the sedentary mice ($p=.003$; $f=7.8$). Post hoc analysis indicated the tumor mass was significantly smaller in wheel running mice given indomethacin than in the other two groups ($p=.003$) See figure 3.

## Discussion

This study found that indomethacin did not preserve muscle mass in tumor-bearing mice or improve VWRA in tumor-bearing mice. Previous studies using this model of tumor-induced muscle wasting did find indomethacin to preserve muscle mass. The dose of the drug used in the pervious studies (5mg/kg/day) for sedentary mice housed in groups was found to be toxic in the mice housed individually given running wheels in this study. The dose was reduced by half for the individually housed mice on wheels (2.5mg/kg/day) and still found to be toxic as shown by the reduced VWRA. Other NSAIDs known to reduce muscle wasting in tumor-bearing mice need to be studied to determine their effects on VWRA.

In the present study, muscle mass was not significantly reduced in tumor bearing mice compared to the control mice. This may reflect the short duration of the study compared to prior

studies using the model (17 days verses 21 days of tumor growth). Also, the effect of the mice being housed individually could be stressful for this species of rodent.

In this study, it was found that both tumor bearing mice and mice treated with indomethacin had a decline in VWRA during the dark phase of the running cycle over the last 15 days. VWRA was reduced in both control mice treated with indomethacin and tumor-bearing mice with indomethacin. Indomethacin did not maintain VWRA in tumor bearing mice in this study. In this study, we found a significant negative correlation between tumor mass and VWRA. Mice with larger tumors were found to have decreased VWRA during the last three days of the experiment compared to mice with smaller tumors. Also, the study found a significant positive correlation between average gastroc muscle weight and VWRA. Mice with larger gastroc muscles were found to run more during the last three days of the experiment than mice with smaller gastroc muscles. A significant negative correlation between tumor weight and muscle mass (gastroc relative to body weight) was also found in this study suggesting the larger the tumor the smaller the muscle mass.

Limitations of this study include housing mice individually compared to the usual group sedentary housing.

## Conclusion

This study found 2.5mg/kg/day indomethacin, a dose normally tolerated by sedentary tumor-bearing mice, to be toxic in mice housed individually and given unlimited access to running wheels. Indomethacin did not preserve muscle mass or maintain VWRA in tumor bearing mice. More research is needed to determine if other NSAIDs would preserve muscle mass and VWRA in this or other models of tumor-induced muscle wasting and fatigue.

Curiously, tumor growth was significantly reduced in mice with access to both running wheels and indomethacin, compared to sedentary mice, or running mice not given indomethacin. Further studies are needed to explore the significance of this finding.

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# Appendix

Figure 1: Muscle Mass by Group

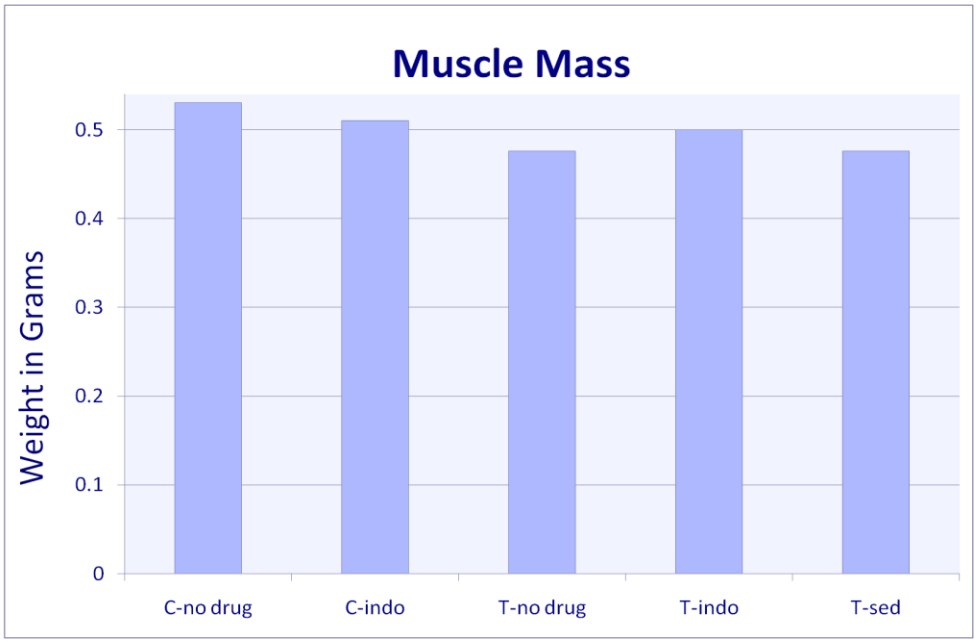


Figure 2: VWRA Over Time by Group

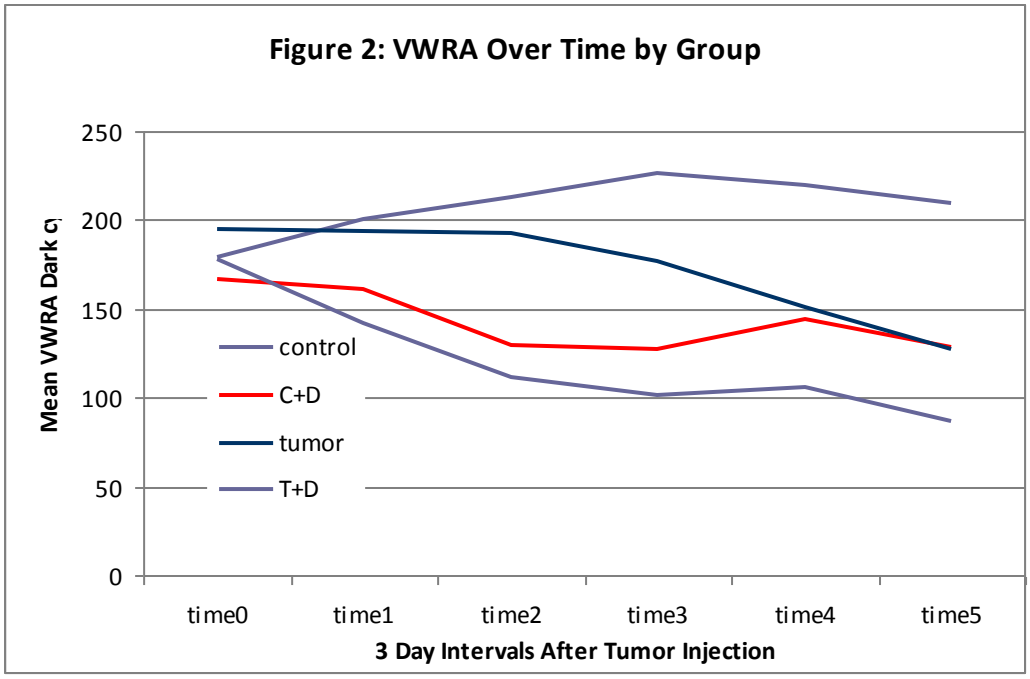

Figure 3: Tumor Mass in C26 Mice by Group